Effects of vasoactive agents in healthy and diseased human saphenous veins

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Purpose: **Smooth muscle reactivity is one of the factors involved in the pathogenesis of varicose veins. We investigated the myotropic effects of the 3 main vasoconstrictor agents—norepinephrine (NE), angiotensin II (Ang II), and endothelin-1 (ET-1)—in isolated human saphenous veins.**

Methods: **Human saphenous veins were collected from 23 patients with primary chronic venous insufficiency who underwent elective varicose vein resections and who were stratified into the following 3 groups: group 1, 7 patients in clinical class 2; group 2, 9 patients in clinical classes 3 and 4; and group 3, 7 patients in clinical classes 5 and 6. Moreover, 6 patients who underwent arterial bypass grafting procedures represented the control group. The tissues were suspended in organ baths that contained Krebs solution, and their mechanical responses were measured isometrically. The cumulative concentration–response curves to Ang II, NE, and ET-1 were performed at 90-minute intervals in each tissue.**

Results: **In the control tissues, NE, Ang II, and ET-1 induced concentration-dependent contractions with apparent affinities (pEC_{50}, the negative logarithm to base 10 of the molar concentration of the agonist, which produces the 50% of the maximal effect) and maximal effects (maximum effect, g of contraction) that were equal to 7.06 ± 0.23, 8.53 ± 0.34, 7.63 ± 0.10, and 2.21 ± 0.33, 1.65 ± 0.31, 2.60 ± 0.77, respectively. Two main findings were evident in comparison of varicose veins with control tissues. First, the maximum effect that was evoked by all of the stimulants was reduced progressively with the increasing severity of the disease, which raised the third group to statistical significance for both NE and Ang II ($P < .05$). Second, a marked reduction of Ang II apparent affinity was already evident in tissues that were taken from patients in an early stage of the disease ($P < .05$).**

Conclusion: **The demonstration of a significant reduction in Ang II and NE contractile activities and the important reduction of that of ET-1 in the diseased veins as compared with the control tissues extends the previous observations regarding the impairment of smooth muscle contractility in primary chronic venous insufficiency. Moreover, the dramatic reduction of Ang II affinity, which appears in an early stage of the disease, supports the hypothesis that such abnormality within the venous wall could play a role in the pathogenesis of primary varicose vein disease. (J Vasc Surg 1998;28:855-61.)**

Varicose veins are the most frequent manifestations of chronic venous insufficiency (CVI), which increases in incidence with age and whose estimated rate of occurrence can vary worldwide from 10% to 50%. The dilatation of veins appears to be a primary process that is initiated by unknown factors.[1] There are several theories about the possible mechanisms that initiate the disease process.[2] One of the more often evoked theories concerns the weakness of the venous wall, which in turn leads to the separation of the valve cusps with consequent blood reflux. By acting on the vein wall, this hemodynamic change, and particularly high reverse flow velocity and turbulence, facilitates an extensive tissue remodeling.[2-6]

In support of this hypothesis, evidence is accumulating that implicates a deficient smooth muscle function in the pathogenesis of CVI. For instance, it was reported that the maximal responses of a varicose vein preparation to norepinephrine (NE) and to endothelin-1 (ET-1) are lower than those of control

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Table I. Characteristics of the selected patients divided into 3 groups of increasing severity of disease on the basis of the CEAP classification

	C		*E*		*A*		*P*	
Group 1	C2s	7 patients	Primary	7 patients	S2, P17	5 patients	Reflux	7 patients
					S2-S3, P17	1 patients		
					S2-S3, P17-18	1 patients		
Group 2	C3s	7 patients	Primary	9 patients	S2, P17	1 patient	Reflux	9 patients
	C4s	2 patients			S2-S3, P17	2 patients		
					S2-S3, P17-18	6 patients		
Group 3	C5s	5 patients	Primary	7 patients	S2-S3, P18	2 patients	Reflux	7 patients
	C6s	2 patients			S2-S3, P17-18	5 patients		

C, Clinical; *E,* etiologic; *A,* anatomic; *P,* pathophysiologic.

healthy tissues.[3,7-9] Whether these functional changes cause a multifocal impairment of vein smooth muscle and mediate the development of varicose veins or merely represent an epiphenomenon of the pathophysiologic process still remains a contentious issue.

The aim of the present study was the comparison of the myotropic effects of the 3 main endogenous vasoconstrictor agents—NE, ET-1, and angiotensin II (Ang II)—along the natural history of primary varicose veins disease (PVV). The effects of these agents were tested in saphenous vein tissues that were obtained both from patients with PVV of different clinical classifications and from subjects without any evidence of coexistent venous disease (the control group). The testing was performed to reconstruct the stage in which the changes in contractile response become evident along the progressive course of the disease.

MATERIALS AND METHODS

Patient population. The proximal portion of the greater saphenous vein (GSV) was obtained from 23 patients (7 men, 16 women; mean age, 51 ± 11 years) who were referred to our vascular laboratory for PVV and from 6 patients (6 men; mean age, 62 ± 6 years; controls) who underwent a limb salvage procedure with in situ saphenous bypass grafting. The patients for varicose veins with a competent proximal tract of the GSV, with post-phlebitic and congenital venous disease, were excluded from the present study. The selection was made in accordance with the clinical and duplex criteria stated below. The patients for PVV were separated into 3 distinct clinical groups of increased severity of the disease on the basis of the clinical, etiologic, anatomic, and pathophysiolobic (CEAP) classification. The CEAP classification is summarized in Table I.[10]

The diagnosis of varicose vein disease was made with the combination of clinical examination (positive Trendelenburg's test and Perthes' test) and duplex scanning examination (Ansaldo AU 4, 7.5 to 10 MHz, Esaote Biomedica, Genova, Italy). Superficial vein incompetence was diagnosed when a reverse flow longer than 0.5 seconds[11,12] was detected in at least 1 saphenous segment, with the Doppler scan sample volume placed at an angle of 45 degrees at 5 different levels along the GSV and the saphenofemoral junction. Reflux was evoked through manual calf squeezing in the patient who was standing; the manual squeezing maneuver always was performed by the same investigator (P.Z.). Duplex analysis also investigated the common and the superficial femoral veins. The investigation of the popliteal region included the saphenopopliteal junction, the proximal tract of the lesser saphenous vein, the gastrocnemius vein, and the popliteal vein from the junction level to the opening of the anterior/posterior tibial vein. When an unsatisfactory image of the anterior/posterior tibial vein was obtained, the veins were studied distally. Finally, the perforating vein system was investigated in accordance to the duplex scan methodology that was described by Labropoulos et al.[13]

We also investigated the disease duration in the selected patients, which was self-assessed by the patients through the date of onset of visible varicose veins or of typical symptoms of CVI. Because of the admitted unreliability in the determination of disease duration with a single question, the physicians spent some time in assessing the disease duration with a series of other questions to relate the appearance of CVI symptoms or varicose veins with other precise life events.

The patients gave informed consent, and the protocol was approved by the Ethics Committee of our hospital. The selected patients underwent air-pletysmographic examination (APG)[14,15] for the assessments of total volume, venous filling index (VFI), ejected fraction, and residual volume fraction

(RVF). The APG examination was performed in all of the cases between 8 AM and 10 AM at the same temperature (23°C) before the surgery was performed for the correction of CVI.

Tissue collection. The patients in the control group were affected by critical limb ischemia and did not have any clinical history or evidence of venous disease. All of the patients underwent in situ femorodistal bypass graft procedures, and the strip of the saphenous vein, which was never shorter than 1 cm, was taken from the junction level just before performing the proximal anastomosis. Pathologic saphenous vein samples were obtained during surgery for superficial venous insufficiency. The samples were taken from the junction at the time of saphenofemoral disconnection and carefully excised with direct visualization.

Tissue preparation. After the surgery, the segments of human saphenous vein (hSV) were placed in a cold Krebs solution (4°C). The lapse of time between the surgery and the experiment was 15 hours on the average.

In the preliminary experiments, we tested the contractile responses of the same vein to different agents immediately after the surgery (delay, 0 hours) and after a 24-hour stay in a cold Krebs solution (4°C). No significant differences were found between the 2 sets of data. These data indicated that the lapse of time between the surgery and the experiment did not influence the results of the experiments.

In the laboratory, the tissue was placed in a Krebs solution at room temperature and, within 30 minutes, the vein was dissected free of surrounding tissues. The tissues then were cut into open rings (2 to 4 rings for each vein), mechanically denuded of their endothelium, and suspended in 10-mL organ baths that contained a Krebs solution that was gassed with 95% O_2 and 5% CO_2 (pH, 7.4). The strips were preloaded with 1 g. The mechanical responses were measured isometrically by Grass FT03 force transducers (Selb, Germany) and recorded on a Linseis multichannel chart recorder (model 2005, Selb, Germany).

Experimental protocol. After an equilibration period of about 2 hours, the preparations were contracted with KCl (100 mmol/L, standard stimulus). The absence of relaxation to ACh (1 μmol/L) in tissues that were precontracted by NE (1 μmol/L) indicated the absence of a functioning endothelium.[16,17] The cumulative concentration–response curves (CRCs) to Ang II, NE, and ET-1 were performed at 90-minute intervals in each tissue. The CRCs were obtained with the application of increasing concentrations of agonists (0.5 logarithm unit steps). The tissues did not loose contractility for 5 to 6 hours of in vitro incubation. This was established in the preliminary experiments when we did not find significant differences between the contractile effects of 100 mmol/L KCl applied at the start and at the end of the incubation period. This also was observed in the tissues that were used to record CRCs to Ang II and NE. All of the in vitro experiments were performed by investigators who were unaware of the clinical assessments of the patients from whom the tissues were collected.

Materials. ET-1, Ang II, substance P, and bradykinin were purchased from the American Peptide Company (Sunnyvale, Calif). All of the other substances and reagents were from the Sigma Chemical Company (St Louis, Mo) or from E Merck (Darmstadt, Germany). The stock solutions (1 mmol/L) were made in distilled water and kept at –20°C until use. The Krebs solution had the following composition (in mmol/L): NaCl, 118.5; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; and glucose, 10.

Data analysis and terminology. All data are expressed as the mean ± the standard error of the mean. The difference in disease duration and in venous function among clinical classifications were tested for significance with 1-way analysis of variance followed by the Student-Newman-Keuls test for multiple comparison with a software package.[18] Pharmacologic data were statistically analyzed with 1-way analysis of variance followed by the Dunnett test for multiple comparison with a software package.[18] *P* values that were lower than .05 were considered to be significant. The pharmacologic terminology that is adopted in this paper is in accordance with the recent recommendations of the International Union of Pharmacology Committee on Receptors Nomenclature and Drug Classification.[19] The agonist apparent affinities are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect.

RESULTS

The patients with PVV were classified in accordance with the new CEAP classification criteria[10] that are summarized in Table I. The following classifications were seen: 7 patients had noncomplicated but symptomatic varicose veins (clinical class, C2s; first group of 7 patients); 7 patients had varicose veins that were complicated by edema (clinical class, C3s): and 2 patients had edema and skin changes (clinical class, C4s): these classifications constitute the second group of 9 patients; 7 patients had healed

Table II. Results of air-plethysmographic examination determinations and of assessment of disease duration in patients with primary varicose vein disease stratified into 3 clinical groups of increasing severity

	Total volume (ml air)	*Venous filling index (ml air/min)*	*Ejection fraction (% of TV)*	*Residual volume fraction (% of TV)*	*Disease duration (years)*
Group 1 (clinical C2)	109 ± 36	2.2 ± 1.4	57 ± 21	23 ± 12	7 ± 4
Group 2 (clinical C3 to C4)	163 ± 50*	5.6 ± 1.6*	53 ± 9	33 ± 14	18 ± 6*
Group 3 (clinical C5 to C6)	177 ± 19*	6.2 ± 0.8*	48 ± 4	46 ± 3*	21 ± 3*
Normal values	<150	<1.7	>70	<33	

*P < .05 versus group 1.

Table III. Effects of KCl, norepinephrine, angiotensin II, and endothelin-1 in human saphenous vein strips from control subjects and from patients with primary varicose vein disease in 3 groups of increasing severity of the disease

		Norepinephrine		*Angiotensin II*		*Endothelin-1*	
	KCl 100mM	*pEC_{50}*	*Emax (g)*	*pEC_{50}*	*Emax (g)*	*pEC_{50}*	*Emax (g)*
Group 1 (clinical C2)	1.44 ± 0.29	6.68 ± 0.27	1.96 ± 0.35	7.63 ± 0.29*	1.44 ± 0.27	7.53 ± 0.56	2.29 ± 0.42
Group 2 (clinical C3 to C4)	0.94 ± 0.23	6.54 ± 0.28	1.69 ± 0.32	7.42 ± 0.20*	0.74 ± 0.17	7.32 ± 0.51	2.02 ± 0.68
Group 3 (clinical C5 to C6)	0.44 ± 0.14†	6.81 ± 0.19	0.97 ± 0.16*	7.46 ± 0.27*	0.45 ± 0.15*	7.64 ± 0.45	1.02 ± 0.28‡
Control tissues	1.16 ± 0.28 g	7.06 ± 0.23	2.2 ± 0.33	8.53 ± 0.34	1.55 ± 0.31	7.63 ± 0.10	2.40 ± 0.57

*P < .05.
†P = .056.
‡P = .076 vs control group.

or active ulcers (clinical class, C5s to C6s; third group of 7 patients).

In all of the cases, the etiology was primary (E_p). The anatomic (A) distribution of the disease is detailed in Table I. Venous insufficiency confined in the superficial system of the above-knee GSV territory is indicated by S2 and that of the below-knee by S3. P17 and P18 indicate perforators insufficiency of the thigh or calf territory, respectively. The pathophysiology (P) of the disease was always as a result of reflux. The following algorithm describes the patients who are included in the present study:

C, 2s to 6s; E_p; A, S2 to S3; P17 to P18, Pr.

APG indices that were measured for all of the patients (and divided in accord to clinical classifications and the disease duration, subjectly determined as above described) are given in Table II.

The first group of patients appeared to be in an early stage of the disease. This is shown by the significant differences that are observed in the total volume, the VFI, and the RVF values between group 1 on one side and groups 2 and 3 on the other.

Functional in vitro assays showed that 1 mmol/L of ACh, applied in veins that were previously contracted with 1 mmol/L NE, did not induce any relaxation. Similar results also were obtained with bradykinin or substance P (both 100 nmol/L) as vasorelaxation agents (data not shown). This data confirmed the absence of functional endothelium in the strips.

In control tissues, 100 mmol/L of KCl induced a rapid contraction that reached a plateau at 1.16 ± 0.28 g. In the same tissues, cumulative CRCs to NE, Ang II, and ET-1 were performed at 90-minute intervals. The 3 vasoconstrictor compounds induced concentration-dependent contractions of hSV strips. The contractions that were induced by NE and Ang II were rapidly reversible after washing, and those that were evoked by ET-1 were not reversible. For this reason, the CRCs to ET-1 always were performed at the end of the experiments.

A comparison of the contractile effects of KCl, NE, Ang II, and ET-1, which were obtained in saphenous veins from patients with PVV, and control is presented in Table III. The apparent affinities of the agonist, which were expressed in terms of pEC_{50} and maximal contractile responses (maximum effect [Emax], measured in g), have been used to evaluate the effects of these substances on smooth muscle fiber contractility. The data obtained with KCl, the depolarizing nonspecific agent, indicate

that the pathologic lesions of the veins reduce the strip contractility ($P = .056$ in group 3, exactly at the limit of significance).

NE shows little if any changes of apparent affinity, but the maximal contractile response to the catecholamine is significantly reduced in the tissues of group 3 ($P < .05$). Similar results, although not statistically significant, were obtained with ET-1, the potent long-acting vasoconstrictor. The affinity of this agonist is similar in the 4 groups of tissues, and maximal contractility is slightly lower in the tissues from group 2 but is reduced by half in patients of group 3 ($P = .076$). The contractile responses to Ang II were reduced in the tissues from all of the PVV patients. The maximal effect that was induced by the octapeptide was one half and one third of the control values in the vein of groups 2 and 3, respectively—the values of the latter being significantly different from the values of the control group. In addition, a dramatic reduction (more than 10-fold) of Ang II pEC_{50} was observed between the control and the diseased tissues. Interesting enough, the reduction of Ang II apparent affinity already was evident and statistically significant in the tissues of group 1 as compared with the control group. Moreover, in the diseased veins, the application of Ang II induced, simultaneously with the increase of tension, spontaneous activity of the tissues (rhythmic spikes). This phenomenon was never observed in the control tissues or in the diseased tissues that were stimulated by NE and ET-1. The results that are summarized in Table III are illustrated in Fig 1 by showing the mean ± the standard error of the mean of the CRCs for NE, Ang II, and ET-1 in the control and the diseased tissues.

DISCUSSION

The maintenance of the venous tone is expected to be largely controlled by the sympathetic nerves, by the autacoids, such as the endothelins, and by the vasoactive hormones, such as angiotensin.[3,7-9,20-23] The results that are presented above show that all of these stimulants are capable of inducing concentration–dependent contractions in hSV tissues by acting through different mechanisms: KCl 100 mmol/L, by inducing cell membrane depolarization; and NE, ET-1, and Ang II by activating specific receptors that are located in the smooth muscle cells of the hSV. It has been shown that in this preparation Ang II acts via angiotensin receptor-1 receptors,[22] and NE and ET-1 exert their contractile activities by activating a mixed population of $\alpha 1$-$\alpha 2$[21] and ET_A and ET_B receptors,[8,23] respectively.

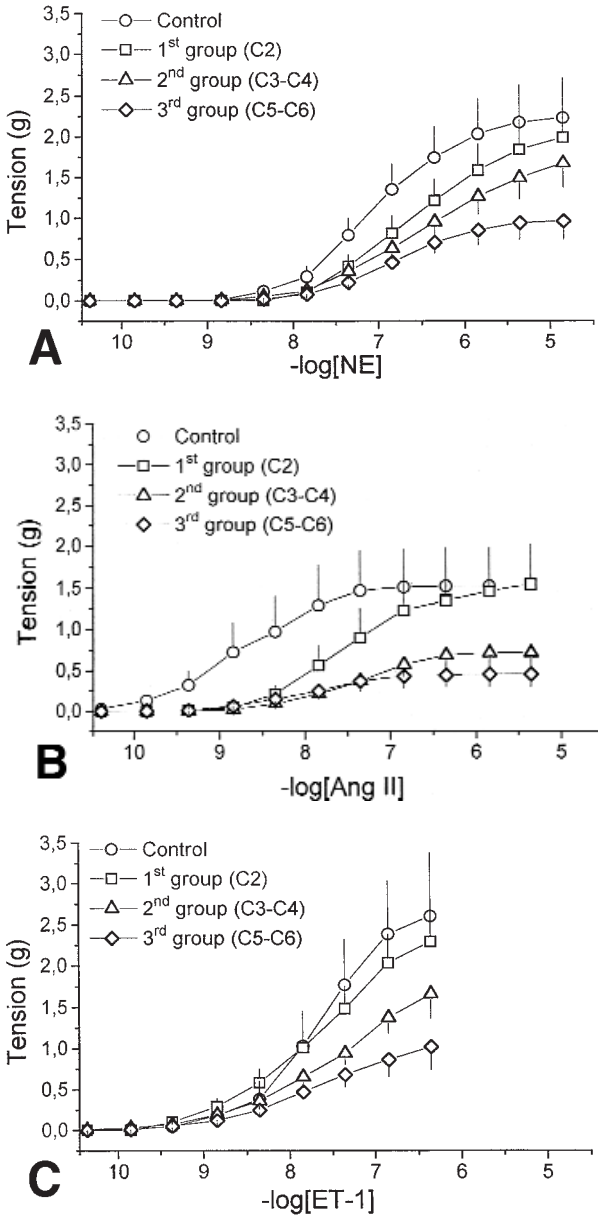

Fig 1. A, Concentration response curves to norepinephrine *(NE)*, **B,** angiotensin II *(Ang II)*, and **C,** endothelin 1 *(ET-1)* in human saphenous veins from control subjects and from patients with primary varicose vein disease. *Abscissa*, logarithm of the molar concentration of agonist; *ordinate*, tension in g.

The values of apparent affinity (pEC50) that are measured in the control tissues (7.06, 8.53, and 7.63 for NE, Ang II, and ET1, respectively) are close to those that are obtained with similar experimental conditions by other authors—NE 7.25,[21] Ang II 8.61,[22] and ET-1 8.06.[23] Thus, the present data are in concord with the current literature.

The comparison between the responses of the control tissues and those that were obtained in diseased tissues indicates that the following two different phenomena occur during the natural history of PVV: first, a nonspecific loss of contractility of vein strips, which appears only in the late phases of the disease; and second, a dramatic decrease of sensitivity to Ang II, which occurs in an early stage of PVV.

The loss of contractility of the PVV strips is documented by the reduction of Emax induced by NE, Ang II, and ET-1 and by the reduction of the contractile effect of 100 mmol/L KCl. Moreover, this decrease in contractility is not present in group 1 (clinical class C2) but becomes evident in group 2 (C3 to C4) and important in group 3 (C5 to C6), where the level of significance is raised for both NE and Ang II. In addition, the important decline of contractility in response to ET-1, although not significant ($P = .076$), confirms the progressive worsening of the vein wall properties with the increasing severity of the disease.

These results can be easily interpreted on the basis of the well known remodeling phenomenon. In the comparison of normal and varicose vein walls, both the histologic and biochemical data showed an extensive tissue remodeling, with the latter exhibited by increased levels of plasminogen activator.[24] The most prominent histologic lesions that can be seen in varicose veins is an increase in the fibrous connective tissue of the media. There is also the separation of the smooth muscle cells from one another, and the elastic fibers tend to spread throughout the vein wall instead of being confined to the internal or external elastic lamina. These changes are generally patchy: electron microscopy of smooth muscle cells shows them to contain collagen fibers, which suggest that the smooth muscle cell has assumed phagocytic properties. A decrease in the total smooth muscle content also has been shown and is considered to be one of the signs of the disease progression within the vein wall in varicose conditions.[4-5,8,24-26]

The functional changes that we attempted to evaluate in the present study with vasoconstrictors are in accordance with the histologic picture of remodeling, which is outlined above. In fact, Emax is expected to provide a rough measurement of muscle receptor numbers, and pEC_{50} reflects the interaction of the agent with the receptor and gives an estimate of individual receptor function. The results of the present investigation clearly indicate that Emax is reduced for all vasoconstrictors and that the reduction is progressively more evident with the gravity of the disease. In group 3, Emax: 1), reaches the level of significance for NE and Ang II; 2), is at the limit of significance for KCl; and 3), is less than half of the control values for ET-1.

Thus, the remodeling of the muscle layer may impair the vasoactive agents–mediated contractility and appears to be reflected by the decrease in receptor numbers and Emax, especially in the late phases of the disease. Conversely, the receptors appear to interact efficiently with the vasoconstrictors, at least with NE and ET-1, because the pEC_{50} values for these agents do not change with the severity of the disease.

The present results confirm the findings of a recent study by a group of investigators from the Mayo Clinic. By measuring receptor binding, Barber et al[9] found that the number but not the affinity of ET-1 receptors was diminished in the tissues of PVV. They concluded that, as the veins become varicosed, there is a specific loss of receptors for ET-1.

On the contrary, the decrease in the apparent affinity for Ang II is already present in the tissues from group 1 and is found in the other groups without further aggravation. The patients of group 1 had a short disease duration and had no significantly altered venous function as shown in Table II. In fact, the mild intensity of reflux that was expressed by the VFI index shows that little hemodynamic changes have occurred. In addition, the acceptable calf pump function, expressed by ejected fraction and partially by RVF values, suggests that no extra vascular factors are involved in the pathogenesis of CVI in this group of patients. Therefore, neither clinical nor in vitro signs of extensive remodeling are evident.

The decrease of affinity, specific for Ang II, may be interpreted by assuming that, in diseased tissues, changes occur at the Ang II receptor level. These receptors become more than 10-fold less sensitive to the agonist and their ability to induce contraction is not changed (the Emax in control tissues and in tissues of group 1 is not different). Such changes cannot be explained by a reduced accessibility of Ang II to its receptor because tissues of group 1 show little if any signs of remodeling. Accentuated local enzymatic degradation of Ang II seems unlikely, but no data are available to exclude this possibility. The intracellular smooth muscle machinery that subserves contraction appears to be efficient because NE and ET-1 and also high concentrations of Ang II evoke maximal contractions. The alteration is therefore to be placed at the level of the Ang II–receptor interaction. The present data cannot clarify whether such an alteration is just an epiphenomenon or is a primary defective mechanism (probably genetically

determined) that is involved in the pathogenesis of the disease. Worthy of mention is the familial distribution of the disease: Cornu-Thenard et al[27] found that the risk of suffering from PVV for children was 90% when both parents were affected, 25% for male patients and 62% for female patients when one parent had this disease, and only 20% when neither parent was affected.

Taken together, the present findings support the intriguing hypothesis that a primary defective mechanism in the Ang II–mediated control of the venous tone could play a role in the pathogenesis of CVI. In consideration of the primary role that is played by the renin-angiotensin system in cardiovascular pathophysiology, these findings could have important implications for drug development and therapeutic uses, particularly of angiotensin converting enzyme inhibitors and angiotensin receptor antagonists.

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